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(71) Applicant *(for all designated States except US)*: GILEAD SCIENCES, INC. [US/US]; 344/346 Lakeside Drive, Foster City, CA 94404 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : LATHAM, John, A. [US/US]; 1619 26th Avenue, San Francisco, CA 94122 (US). LIN, Kuei-Ying [-/US]; 4774 Cavasback Common, Fremont, CA 94536 (US). MATTEUCCI, Mark [US/US]; 1524 Columbus Avenue, Burlingame, CA 94010 (US).

(74) Agent: MISROCK, S., Leslie; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

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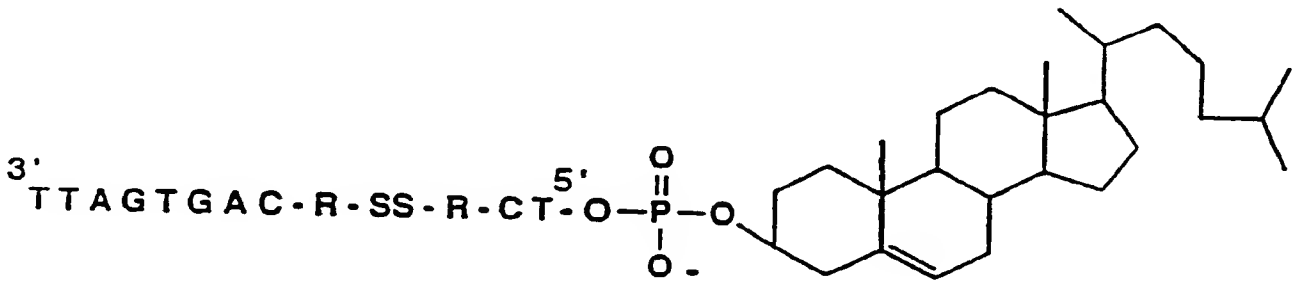

(57) Abstract

The invention relates to compositions and methods for nhancing the delivery of an oligonucleotide into a cell. The compositions of the invention comprise oligonucleotide conjugates which consist of an oligonucleotide, conjugated via a molecular linker containing at least one disulfide bond, to an agent (termed herein "transport agent") which facilitates transport across an outer cell membrane, r across the blood-brain barrier. In a preferred aspect, the disulfide linkage is cleaved upon uptak of the composition by the cell. Pharmaceutical compositions comprising an oligonucleotide conjugate of the invention may be used to treat a wide variety of diseases and disorders. Methods for inhibiting the expression of a nucleic acid sequence within a cell, and methods f r detecting a nucleic acid sequence within a cell are also provided. In a specific embodiment, an oligonucleotide conjugated to cholesterol via a linker containing a disulfide linkage can be used for therapeutic or diagnostic purposes, by hybridization of the oligonucleotide to a complementary nucleic acid sequence in a procaryotic or eucaryotic cell.

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OLIGONUCLEOTIDE-TRANSPORT AGENT DISULFIDE CONJUGATES

1. FIELD OF THE INVENTION

The invention is directed to compositions and methods for enhancing the delivery of an oligonucleotide into a viable cell or organism. The compositions of the invention comprise oligonucleotide conjugates consisting of an oligonucleotide conjugated via a molecular linker containing at least one disulfide bond, to an agent which facilitates transport across a cell membrane, or across the blood-brain barrier. Also included within the present invention are oligonucleotide conjugates containing a molecular linker having at least one disulfide bond wherein the molecular linker confers stability under extracellular conditions but is labile under intracellular conditions. In a preferred aspect, the disulfide linkage is cleaved during or after uptake of the composition into the cell. The invention is also directed to methods for inhibiting the expression of a nucleic acid sequence in a cell comprising providing the cell with an oligonucleotide conjugate of the invention. In a specific embodiment, the oligonucleotide can hybridize to the nucleic acid sequence. Additionally, the invention is directed to methods for detecting a nucleic acid sequence in a cell comprising contacting the cell with an oligonucleotide conjugate of the invention, in which the oligonucleotide can hybridize to the nucleic acid sequence, and in which the nucleic acid sequence is detectably labeled. Furthermore, the present invention also includes a method for detecting the presence of a nucleic acid sequence of an exogenous infectious agent utilizing oligomer-disulfide conjugates in diagnostic probes. In a preferred aspect, the oligomer-disulfide

conjugates utilized in the diagnostic probe includes covalent crosslinking agents, which results in increased sensitivity and reduced background in diagnostic or detection assays. Pharmaceutical compositions and therapeutic methods are also provided.

2. BACKGROUND OF THE INVENTION

2.1. CONJUGATION OF MOLECULES VIA A DISULFIDE LINKAGE

Methods for the preparation of protein conjugates via an intermolecular disulfide exchange reaction in which protein A containing thiol groups reacts with protein B containing 4-dithiopyridyl groups to yield a conjugate with the release of 4-thiopyridone has been disclosed (King et al., 1978, Biochemistry 17:1499-1506). It was suggested that coupling of a protein antigen to different protein carriers can be used to enhance or suppress the immunogenicity of an antigen and that conjugates of peptide hormone with a protein carrier may be useful for elucidating hormone-receptor interactions.

Methods have also been disclosed for linking oligonucleotides to nucleic acids, proteins, and thiol-specific fluorescent probes via disulfide bonds (Chu and Orgel, 1988, Nucl. Acids Res. 16:3671-3690; Zuckerman et al., 1987, Nucl. Acids Res. 15:5305-5321; Connolly, 1985, Nucl. Acids Res. 13:4485-4501; and Cheng et al., 1983, Nucl. Acids Res. 11:659-669). Such disulfide linkages were generally incorporated by an intermolecular disulfide exchange reaction. Specifically, an adduct containing an S-S linked moiety was exchanged with free thiol groups on another molecule to give the desired disulfide. It has been suggested that such oligonucleotides linked to proteins or fluorographic or chromogenic probes via a disulfide linkage may be used to assay the extent of hybridization between the oligonucleotide and a complementary sequence in vitro (Chu and Orgel, 1988, Nucl. Acids

Res. 16:3671-3690; Zuckerman et al., 1987, Nucl. Acids Res. 15:5305-5321; Connolly, 1985, Nucl. Acids Res. 13:4485-4501; and Cheng et al., 1983, Nucl. Acids Res. 11:659-669).

Recently, a conjugate containing an oligonucleotide linked to staphylococcal nuclease via a disulfide linkage was disclosed (Corey et al., 1989, Biochemistry 28:8277-8286; Corey and Schultz, 1987, Science 238:1401-1403). It was shown that this conjugate could act as a hybrid enzyme, specifically cleaving single-stranded DNA at sites adjacent to the oligonucleotide binding site.

A biotinylated mononucleotide or mononucleoside linked to an organic basic group via a chemically cleavable bond, e.g. a disulfide linkage, has also recently been disclosed (Herman, U.S. Patent No. 4,772,691, issued September 20, 1988). It was suggested that such compositions may be useful in isolating target macromolecules from crude physiological mixtures. Specifically, it was suggested that the biotinylated nucleotides may contact a target macromolecule via their organic basic groups to macromolecules having an affinity for the target molecule, resulting in the formation of a biotinylated nucleotide-affinity macromolecule-target macromolecule complex. Such a complex may be brought into contact with immobilized avidin, which binds to the biotin moiety. The nucleotide may be cleaved via the cleavable bond to obtain the affinity-macromolecule-target macromolecule complex from which the target macromolecule may be obtained.

Other publications have reported the disulfide linkage of DNA to another molecule. Chu and Orgel (1988, Nucl. Acids Res. 16:3671) disclose the linkage via cleavable disulfide bonds of, inter alia, a 16-mer oligonucleotide to peroxidase, and a viral RNA to an IgG, and suggest th ir use in detection assays on

a solid phase or, preferably, in solution. Cheng et al. (1983, Nucl. Acids Res. 11(3):659) disclose th disulfide conjugation of G-tailed plasmid DNA (the herpes simplex virus-1 thymidine kinase gene or the E. coli chloramphenicol acetyltransferase gene) to α_2 - macroglobulin (a protein subject to receptor mediated endocytosis).

Within the standard animal cell, glutathione (a cysteine containing peptide) is found in high concentration in the reduced form (~1-5 mM) (reviewed in Meister and Anderson, 1983, Ann. Rev. Biochem. 52:711-760). This free thiol has powerful redox activity towards cleaving disulfides to thiols independent of their composition.

2.2. TRANSPORT OF AGENTS INTO CELLS

It is widely accepted that the usefulness of many therapeutic agents is dependent on their ability to be taken up by their target cell population (reviewed in Gregoriadis, 1978, Nature (London) 265:407-411). Problems have been encountered in certain cases of drug resistance development where cells have become impermeable to drugs and where antimicrobial drugs fail to enter intracellular sites harboring microorganisms.

One approach for effecting drug delivery to a target site involves attaching the drug to a carrier capable of transporting the drug from the site of application directly to the site of action (reviewed in Gregoriadis, 1989, in Drug Carrier Systems, ed. F.H.D. Roerdink and Koon, John Wiley & Sons, Ltd., pp. 1-31). Such carriers may be divided into three categories: (1) linear polymers; (2) cells; and (3) three dimensional systems (e.g. liposomes).

Linear polymers are usually covalently linked to the drug via a hydrolyzable bond (reviewed in Hoes and Feijen, 1989, in Drug Carrier Systems, eds. F.H.D.

Roerdink and A.M. Kroon, John Wiley & Sons, N.Y., pp. 57-109). Once the conjugate is in the target area, the linkage between the drug and the carrier is cleaved effecting the release of the drug. In one approach, the carrier is biodegradable. Examples of such carriers include proteins, which are cleaved by proteolytic enzymes inside the cell, polysaccharides which are cleaved by glycosidases or vinyl polymers, which contain hydrolytically labile ester bonds. The success of this approach is dependent on a number of factors which include the conformation of the polymeric carrier and the stability of the drug-carrier bond. There is also the risk of incomplete cleavage of the drug-carrier bond. Another approach involves linking a non-biodegradable polymer to the drug via a hydrolyzable linkage.

One such example of the conjugation of a drug to a carrier via a covalent linkage involved the conjugation of poly[N-(2-hydroxypropyl)methacrylamide] (polyHPMA) to p-nitroaniline via peptide linkers of varying length. It was found that the rate of release of the p-nitroaniline was dependent on the length of the linker.

Another example involved the conjugation of methotrexate to poly (D-lysine) via a disulfide linkage (Shen et al., 1985, J.Biol. Chem. 260:10905-10908). Incubation of methotrexate conjugated directly to poly(D-lysine) did not have a cytotoxic effect on cell growth. However, addition of the disulfide linkage resulted in the manifestation of such a cytotoxic effect when such conjugates were added to cells.

Cells could potentially be used to deliver a drug to a specific target site. For example, erythrocytes may be useful in delivering agents to the reticuloendothelial system. However cells are limited in both the range of agents which they can carry and target accessibility.

The third approach, i.e. the use of three dimensional systems such as liposomes and microspheres has the advantage of containing agents within a well protected space. However, these systems have the disadvantages of being limited in tissue selectivity and their size-imposed inability to cross most normal membrane barriers.

Wu and Wu (1987, J. Biol. Chem. 262(10):4429-4432) disclose the use of a disulfide conjugate of a sialoorosomucoid (ASOR) and poly-L-lysine, complexed noncovalently to plasmid DNA (pSV2 CAT), for delivery of the marker plasmid DNA into hepatocytes possessing a receptor for ASOR, by receptor-mediated endocytosis.

Hostetler et al. (PCT Publication No. WO 90/00555) disclose lipid derivatives of antiviral nucleoside analogs.

2.2.1. TRANSPORT OF DRUGS ACROSS THE BLOOD-BRAIN BARRIER

Access of drugs to a given target site may also be prevented due to gross anatomical barriers. One example of such a barrier is the blood-brain barrier. The endothelial cells of the brain capillaries possess tight junctions and layers of glial cells closely surround the capillaries. Therefore a barrier containing primarily lipids must be traversed by a drug in order to gain access to central neurons. Consequently, highly lipid-soluble compounds have been found to reach the brain rapidly after administration, whereas more polar compounds penetrate at a much slower rate.

A number of approaches have been investigated for transporting drugs across the blood-brain barrier. One approach has involved the conversion of hydrophilic drugs into lipid-soluble drugs by blocking the hydroxyl, carboxyl, and primary amine groups on the drug with lipid soluble substances (reviewed in PCT

Application Publication No. WO 89/10134, published April 25, 1988). However, it has been observed that the transport of such substances are still relatively slow. Another approach has involved the intra-arterial infusion of hypertonic substances which transiently open the blood-brain barrier to allow passage of hydrophilic drugs (Neuwalt et al., 1980, Ann. Int. Med 93:137-139). Hypertonic substances however are potentially toxic and may damage the blood-brain barrier. Another approach that has been recently been taken involves the use of a chimeric peptides that include a peptide that acts as a neuropharmaceutical agent conjugated, e.g., by disulfide linkage, to a transportable peptide that is capable of crossing the blood-brain barrier at a relatively high rate by receptor-mediated transcytosis (PCT Application Publication No. WO 89/10134, published April 25, 1988).

2.3. USE OF OLIGONUCLEOTIDES AS INHIBITORS OF THE EXPRESSION OF A NUCLEIC ACID SEQUENCE

Several approaches have been taken to use oligonucleotides that are complementary to selected cellular or viral target nucleic acid sequences to modulate the expression of the target nucleic acid sequence. There have been several reports on the use of specific nucleic acid sequences to inhibit viral replication (see for example Goodchild et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:5507-5511; Wickstrom et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:1028-1032; and Kawasaki, 1985, Nucl. Acids Res. 13:4991). However, the poor absorption of unmodified oligomers by cells (Zamecnik et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:4143-4146) and their sensitivity to cellular nucleases and nucleases present in culture medium and serum (Wickstrom, 1986, J. Biochem. and Biophys. Meth. 13:97-102) limits their use both *in vivo* and *in vitro*.

Several laboratories have attempted to develop modified oligonucleotides that are membrane permeable and nuclease resistant. One approach involves the development of nonionic oligonucleotide analogs. Examples of such analogs include methylphosphonates (Smith et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:2787-2791; Agris et al., 1986, Biochemistry 25:6268-6275; Jayaraman et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1537-1541); phosphorothioates (Agarwal et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 83:4143-4146; Matsukura et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:7706-7710; Marcus-Sekura et al., 1987, Nucl. Acids Res. 15:5749-5763); and phosphoramidates (Agarwal et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 83:4143-4146).

It has been speculated that phosphorothioates may in addition to binding to complementary target nucleic acid sequences also direct the inhibition of primer binding to HIV reverse transcriptase (Matsukura et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:7706-7710). Antitemplate inhibition has also been described for polymerases using polynucleotides, including partially thiolated polycytidylic acid (reviewed in Stein and Cohen, 1988, Cancer Res. 48:2659-2668).

Another approach has involved conjugating the oligonucleotide to a molecule that will increase the efficiency of uptake of the oligonucleotide by the cell. Examples of such conjugates include cholesteryl-conjugated oligonucleotides (Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556) and a poly-L-lysine conjugate (Lemaitre et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:648-652). Another example includes an oligonucleotide joined through a linking arm to a group that imparts amphophilic character to the final product in order to increase the efficiency of membrane transport (PCT Publication No. WO 88/09810, published December 15, 1988).

Another approach that has been taken involves the use of reactive oligonucleotides, i.e. antisense oligonucleotides linked to reactive agents that are able to modify the target nucleic acid. One such group of reacting agents are intercalating agents which can bind to the duplex by internal insertion between adjacent base pairs or bind to external nucleoside and phosphate elements respectively. Examples of intercalators that have been attached to oligonucleotides and oligonucleotide analogs include acridine, anthridium, and photoactivatable psoralen (reviewed in Zon, 1988, Pharm. Res. 5:539-549). Another such group of reactive groups coupled to oligonucleotides include metal complexes such as EDTA-Fe(II), o-phenanthroline-Cu(I), or porphyrin-Fe(II) (reviewed in Krol et al., 1988, BioTechniques 6:958-976). These compounds can generate hydroxyl radicals in the presence of molecular oxygen and a reducing agent. The resulting radicals can cleave the complementary strand following attack on the target nucleic acid backbone. One problem with using such compounds is since such oligonucleotides are reactive, they may be subject to autodegradation.

2.4. TRIPLE-HELIX FORMATION

Both purine oligodeoxyribonucleotides and pyrimidine oligodeoxyribonucleotides have been observed to bind to double stranded DNA (Griffin and Dervan, 1989, Science 245:967-971). Purine oligonucleotides have been shown to bind antiparallel to purines in duplex DNA by triple helix formation (Beal and Dervan, 1991, Science 251:1360-1363).

Pyrimidine oligonucleotides 15 to 18 nucleotides have been shown to bind with sequence specific dependence to homopurine sites in duplex DNA (Moser and Dervan, 1987, Science 238:634-650). These oligonucleotides bind in the major groove, parallel to the purine strand of Watson-Crick doubl -helical DNA.

The binding affinity and specificity of the pyrimidine oligonucleotid for duplex DNA has been shown to be sensitive to pH, organic cosolvent, added cations, and temperature.

It has been suggested that the sequence specificity of homopyridine oligonucleotides would render such oligonucleotides useful as tools for mapping chromosomes when equipped with DNA cleaving moieties (Moser and Dervan, 1987, Science 238:645-650). Micromolar concentrations of homopyrimidine oligodeoxyribonucleotides have also been shown to block recognition of double helical DNA by prokaryotic modifying enzymes and a eukaryotic transcription at a homopurine target site (Maher et al., 1989, Science 245:725-730). Recently, results of a study of 20 base triplets indicate that the triple helix can be extended from homopurine to mixed sequences (Griffin and Dervan, 1989, Science 245:967-971).

3. SUMMARY OF THE INVENTION

The compositions of the present invention comprise oligonucleotide conjugates. These oligonucleotide conjugates consist of an oligonucleotide conjugated, via a molecular linker consisting of at least one disulfide bond, to an agent (termed herein "transport agent") which facilitates transport across an outer cell membrane and/or across the bood-brain barrier. The compositions of the present invention also comprise oligonucleotide conjugates containing a molecular linker having at least one disulfide bond wherein the molecular linker confers stability under extracellular conditions but is labile under intracellular conditions. In a preferred aspect, the disulfide linkage of the molecular linker is cleaved during or after transport of the composition into the cell. In a specific embodiment of the invention, the oligonucleotide portion of th conjugate consists of at

6-50 nucleotides, with a size of 8-30 nucleotides most preferred. In a preferred embodiment of the invention, the oligonucleotide portion of the conjugate consists of 6-50 nucleotides, and is capable of hybridizing to at least a portion of a nucleic acid sequence within the target cell. The transport agent may be selected from the group including but not limited to cholesterol, a peptide, a protein, a lipid, a saccharide, a nucleoside or analog thereof, an antibody, and a biocompatible polymer. In a specific embodiment, the transport agent is cholesterol.

The invention further provides pharmaceutical compositions comprising an effective amount of the oligonucleotide conjugates of the invention in a pharmaceutically acceptable carrier. Methods for treatment of various diseases and disorders comprising administering the pharmaceutical compositions of the invention are also provided. The invention is thus directed to therapeutic methods involving increased delivery of a therapeutically effective oligonucleotide into a cell, comprising providing the cell with a composition comprising the oligonucleotide conjugated to a transport agent via a molecular linker containing at least one disulfide linkage.

In another embodiment, the invention is directed to methods for inhibiting the expression of a nucleic acid sequence in a procaryotic or eucaryotic cell comprising providing the cell with an effective amount of a composition comprising an oligonucleotide conjugate of the invention. In one embodiment, the expression of the nucleic acid sequence in the cell is inhibited by hybridization of the oligonucleotide with the nucleic acid sequence in the cell. In another embodiment, such composition may inhibit the expression of a nucleic acid in a cell by inhibiting the action of polymerases in the cell. In yet another embodiment, such composition may inhibit the expression of a

nucleic acid sequence in a cell by forming a triple helix with a double-stranded nucleic acid sequence in the cell. In particular, the compositions can be effective antiviral, antifungal, or antibacterial agents. Additionally, these compositions may be used to inhibit the expression of cellular genes such as cellular oncogenes.

The invention is also directed to methods for detecting a nucleic acid sequence within a procaryotic or eucaryotic cell comprising providing a viable cell with a composition comprising an oligonucleotide conjugate of the invention, in which the oligonucleotide thereof is (a) linked to a detectable label, and (b) is capable of hybridizing to the nucleic acid sequence within the cell.

The invention is also directed to methods for detecting a nucleic acid sequence utilizing oligonucleotide-disulfide conjugates in a diagnostic probes comprising an indirect binding assay whereby human body fluids, cell or tissue extracts are screened for the presence of endogenous or exogenous target DNA of a pathogen or gene sequence associated with a pathological state whereby an oligonucleotide probe containing a reporter group linked to the oligonucleotide via a disulfide linker can be added to the material containing the target DNA sequence and the reporter group may be released and measured from the hybrid target/probe DNA complex by adding reducing agents.

The indirect binding assay can be modified such that the oligonucleotide probe containing a linker group with a free thiol group can be hybridized with DNA bound on to a support membrane. A reporter group containing a free thiol group can then be attached to the free thiol group of the oligonucleotide linker complexed with the target DNA, by formation of a disulfide linkage under oxidizing conditions. If

desired, the reporter group target DNA linkage can be subsequently cleaved by exposure to reducing conditions, followed by detection of the reporter group.

Oligonucleotides in diagnostic detection assays, as well as conjugated oligonucleotides for therapeutic use, can be further modified in order to increase sensitivity and reduce background through the incorporation of hybridization-triggered specific crosslinking agents (see e.g., Meyer et al., 1989, J.Am. Chem. Soc. 111:8517-8519; Birg et al., 1990, Nucl. Acids Res. 18:2901-2907; Uhlmann and Peyman, 1990, Chemical Reviews 90(4):543).

Another modification of the above disclosed indirect binding assay involves utilizing probes containing reporter groups and disulfide linkers in combination with nucleic acid analogs that allow sequence specific binding to double stranded DNA bound to a membrane, thereby forming triple helix structures.

The indirect binding assay can additionally be modified utilizing double stranded oligonucleotides (containing the binding site sequence recognized by a cognate DNA-binding protein) to bind specifically to and isolate DNA binding proteins. Additionally, the assay can be modified to use commercially available solid support resins derivatized with thiol groups which will then be used to attach to thiol groups on oligonucleotides by formation of a disulfide bond followed by subsequent binding of a DNA binding protein to the immobilized target DNA sequences.

3.1. DEFINITIONS

An "oligonucleotide" as defined herein is a DNA or RNA sequence comprising at least 6 nucleotides, with an upper limit of about 50 nucleotides. The oligonucleotide may be single-stranded or double-stranded. The oligonucl otide may be modified at the

base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may also include other app nding groups such as peptides. Portions of the phosphate backbone may be replaced by other moieties.

A "peptide" is a fragment of a protein containing at least one amino acid. The peptide may be modified at any reactive site, e.g. amide linkage, and at one or more of the amino acids in the peptide. The peptide may also include other appending groups. The term "oligonucleotide disulfide conjugate" as used herein means an oligonucleotide linked to another entity via a disulfide group. The term "reporter group" as used herein means a entity capable of being detected. The term "reporter group" includes but is not limited to enzymes, fluorescent labels, radioactive labels and biotin avidin labels.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the structure of CHOLESTEROL-TC-R-S-S-R-CAGTGATT.

Figure 2 shows an autoradiogram demonstrating that the disulfide linkage is reduced in oligonucleotide recovered from the cytoplasmic (C) and nuclear (N) fractions of H938 cells incubated with CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTCTCCAT. Samples were recovered 0, 4, and 48 hours of incubation with CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTCTCCAT at 37°C. SM represents starting material, and R represents sample reduced <u>in vitro</u> with 10 mM dithiothreitol (DTT).

Figure 3 shows an autoradiogram of CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTCTCCAT recovered from cell culture medium after incubation of CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTCTCCAT for 0, 4, and 48 hours with H938 cells. SM represents starting material, and R represents sample reduced <u>in vitro</u> with 10 mM DTT. The experiment was run in duplicate.

Figure 4 shows the effect of incubating CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTCTCCAT in RPMI medium + 15% heat inactivated fetal calf serum (FCS) for 0, 30, 60, and 180 minutes. The experiment was run in duplicate. "C" represents control, i.e., compound not incubated in RPMI + 15% heat inactivated FCS.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for enhancing the delivery of an oligonucleotide into a viable cell or organism. The compositions of the invention comprise oligonucleotide conjugates. These oligonucleotide conjugates consist of an oligonucleotide conjugated, via a molecular linker consisting of at least one disulfide bond, to an agent (termed herein "transport agent") which facilitates transport across an outer cell membrane, and/or across the blood-barrier. In one aspect of the present invention, the oligonucleotide conjugates contain a molecular linker having at least one disulfide bond wherein the molecular linker confers stability under extracellular conditions but is labile under intracellular conditions. In a preferred aspect, the disulfide linkage of the molecular linker is cleaved during or after transport of the composition into the cell.

The invention further provides pharmaceutical compositions comprising an effective amount of the oligonucleotide conjugates of the invention in a pharmaceutically acceptable carrier. Methods for treatment of various diseases and disorders comprising administering the pharmaceutical compositions of the invention are also provided.

In another embodiment, the invention is directed to methods for inhibiting the expression of a nucleic acid sequence in a procaryotic or eucaryotic cell comprising providing the cell with an effective

amount of a composition comprising an oligonucleotide conjugate of the invention.

The invention is also directed to methods for detecting a nucleic acid sequence within a procaryotic or eucaryotic cell comprising providing a viable cell with a composition comprising an oligonucleotide conjugate of the invention, in which the oligonucleotide thereof is (a) linked to a detectable label, and (b) is capable of hybridizing to the nucleic acid sequence within the cell.

The invention is also directed to methods for detecting a nucleic acid sequence utilizing oligonucleotide-disulfide conjugates in a diagnostic probes comprising an indirect binding assay whereby human body fluids, cell or tissue extracts are screened for the presence of exogenous target DNA of a pathogenic organism, whereby an oligonucleotide probe containing a reporter group linked to an oligonucleotide via a disulfide linker can be added to the material containing the target DNA sequence, and the reporter group may be released and measured from the hybrid target/probe DNA complex by adding reducing agents.

The indirect binding assay can be modified such that the oligonucleotide probe containing a linker group with a free thiol group can be hybridized to DNA or RNA bound to a support membrane. A reporter group containing a free thiol group can then be attached to the free thiol group of the oligonucleotide linker complexed with the target DNA by formation of a disulfide linkage under oxidizing conditions. If desired, the disulfide linkage may be subsequently cleaved and the reporter group detected.

Oligonucleotides in diagnostic detection assays, as well as conjugated oligonucleotides for therapeutic use, can be further modified in order to increase sensitivity and reduce background through the

incorporation of hybridization-triggered specific crosslinking agents (see e.g., Meyer et al., 1989, J. Am. Chem. Soc. 111:8517--8519; Berg et al., 1990, Nucl. Acids REs. 18:2901-2907; Uhlmann and Peyman, 1990, Chemical Reviews 90(4):543).

Another modification of the above disclosed indirect binding assay involves utilizing probes containing reporter groups and disulfide linkers that also contain nucleotide sequence specific binding to double stranded DNA, thereby forming triple helix structures. For example, such an analog includes, but is not limited to, 5-methylcytosine (Mayer et al., 1989, Science 245:725-730).

The indirect binding assay can additionally be modified utilizing double stranded oligonucleotides (containing the binding site sequence recognized by a cognate DNA-binding protein) to bind specifically to and isolate DNA binding proteins.

Additionally, a binding assay can be carried out using commercially available solid support resins derivatized with thiol groups which will then be used to attach to thiol groups on oligonucleotides to form a disulfide linkage. Subsequent binding of the oligonucleotide to target DNA sequences can be used to isolate such target DNA sequences. The bound complex may then be released from the solid support resin by exposure to reducing conditions.

5.1. OLIGONUCLEOTIDE-TRANSPORT AGENT CONJUGATES

The invention provides compositions for facilitating the uptake of an oligonucleotide by a viable cell. In a preferred embodiment, once taken up by a cell(s) via a transport process, the disulfide linkage within the oligonucleotide conjugate can be cleaved via intracellular reduction, freeing the oligonucleotide from the transport agent. The resulting product is a free thiol which under the high

intracellular r ducing condition is capable of seeking its targ t of interest.

The molecular linker in the conjugates of the invention preferably comprises a hydrocarbon structure containing, at either or both of its termini or internally, at least one disulfide linkage between the oligonucleotide and the transport agent. The molecular linker may contain heteroatoms, an amino acid or peptide, nucleoside or nucleotide, etc. Alternatively, the molecular linker may consist solely of a disulfide. The molecular linker containing one or more disulfide groups can be introduced at either the 5' or 3' terminus of the oligonucleotide, or internally. In specific embodiments, the molecular linker can be introduced at the 5' position of a pyrimidine, the 8' position of a purine, or the 2' position of a sugar within the oligonucleotide.

In another specific embodiment, the molecular linker has the formula:

$$Y\text{-}XCH_2CHR_1\text{-}SS\text{-}CHR_1CH_2X\text{-}Y$$

wherein:

X is O, $\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}$, $\overset{\overset{O}{\|}}{S}$, NR_1, CH_2, $C(R_1)_2$, or $\overset{\overset{O}{\|}}{C}$;

Y is H, Ch_3, alkyl, aryl or $\overset{\overset{O}{\|}}{C}$ when

X = NR_1, CH_2 or $C(R_1)_2$; and

R_1 is H, CH_3, alkyl or aryl.

The above described molecular linker has a controllable $t_{1/2}$ *in vivo*, facilitating its use as a prodrug/transport component. Utilizing these molecular linkers in the oligonucleotide conjugates of the present invention confers stability to the disulfide bond found in the molecular linker under extracellular conditions but allows for cleavag of the disulfide

bond under intracellular conditions. This allows for increased stability of the oligonucleotide conjugate prior to transport into the cell, but provides cleavage of the disulfide bond during or after transport of the composition into the cell.

The regulation, *in vivo*, of the disulfide bond stability is done by varying the groups adjacent to the disulfide bond in the molecular linker. Stability is increased by having election withdrawing groups near the disulfide bond. In a preferred embodiment, X is O or NH_2, Y is $CH_{2CH}2$ or CO, and R_1 is H or CH_3.

Utilizing the above described molecular linker in the oligonucleotide conjugates of the present invention provided a $t_{\frac{1}{2}}$ of >24 hours under extracellular conditions *i.e.*, in a tissue culture medium and a $t_{\frac{1}{2}}$ of ≤1 hour inside a cell. This increased stability under extracellular conditions as opposed to intracellular conditions is shown by a redox potential that is in the range of about -200mV to about -230mV. This is shown in Example 7.8 of the present application.

The oligonucleotide may be conjugated to the transport agent using various procedures known in the art (see for example, Chu and Orgel, 1988, Nucl. Acids Res. 16:3671-3690; Zuckermann et al., 1987, Nucl. Acids Res. 15:5305-5321). In one embodiment, the disulfide linkage may be introduced by reacting the oligonucleotide with a bifunctional reagent containing the disulfide linkage so that the oligonucleotide comprises at least one disulfide linkage. Such reagents include but are not limited to 2-hydroxyethyl disulfide cystamine (Chu and Orgel, 1988, Nucl. Acids Res. 16:3671-3690), 2,2'-dipyridyldisulfide (Chu and Orgel, 1988, Nucl. Acids Res. 16:3671-3690), 1,6-hexanedithiol (Zuckermann et al., 1987, Nucl. Acids Res. 15:5305-5321), 2-iminothiolane (King et al., 1978,

Biochemistry 17:1499-1506), and 6, 6'-hydroxyl hexyldisulfide. The oligonucleotide comprising the disulfide linkage is then reacted with the transport agent containing a free thiol group. Alternatively, a bifunctional agent may be reacted with the transport agent and the resulting product may be subsequently reacted with the oligonucleotide containing a free thiol group, forming a oligonucleotide-transport agent conjugate. Alternatively, a bifunctional reagent which uses disulfide as a junction piece and then is capable of cross-reaction with either proteins or DNA (RNA) can be used. In a specific embodiment, the conjugation methods described in the example Section 6, infra, or a modification thereof, can be used. In another specific embodiment, conjugation can be carried out by use of the reagent N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pierce Chemical Co.) (See e.g., Jung et al., 1981, Biochem. Biophys. Res. Commun. 101:599-606). SPDP readily modifies free-amino groups via formation of an amide linkage. At the same time, this modification introduces an activated disulfide for use in a second reaction involving disulfide exchange. Coupling is achieved via disulfide exchange with an entity containing a free thiol via displacement of 2-thiopyridinone. The resulting product contains the two molecules joined via a reducible disulfide linkage.

5.2. THE OLIGONUCLEOTIDE PORTION OF THE CONJUGATES OF THE INVENTION

The oligonucleotide portion of the conjugates of the invention may be DNA or RNA, single-stranded or double-stranded. In a preferred aspect, the oligonucleotide is single-stranded DNA. In a specific embodiment of the invention, the oligonucleotide portion of the conjugat consists of 6-50 nucleotides, with a size of 8-30 nucleotides most preferred. In a preferred embodiment of the invention, th oligonucleo-

tide portion of the conjugat consists of 6-50 nucleotides, and is capable of hybridizing to at least a portion of a nucleic acid sequence within the target cell. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

The oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil, (acp3)w, and 2,6-diaminopurine.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose,and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et al, 1987, Nucl. Acids Res. 15:6625-6641).

The oligonucleotide may be conjugated to the transport agent via the molecular linker containing a disulfide linkage at the base moiety, at the sugar moiety, and/or at the phosphate backbone of the oligonucleotide.

5.3. THE TRANSPORT AGENTS OF THE CONJUGATES OF THE INVENTION

The transport agents of the invention increase delivery of the oligonucleotides to which they are conjugated to a target cell. The transport agents facilitate transport of the conjugated oligonucleotide across a cell membrane, or across the blood brain barrier in vivo. In a preferred embodiment, the transport agent is cleaved from the oligonucleotide after uptake by the target cell, by intracellular reduction of the disulfide linkage. In the preferred embodiment where a transport agent facilitates entry into a cell across the cell membrane, the transport agent can be a molecule known in the art to gain entry into the cell through adsorptive endocytosis (hydrophobic or electrostatic), receptor-mediated endocytosis, or membrane fusion activity (e.g., as exhibited by liposomes, viral ghost particles, proteins with fusion activity, etc.). Mediators of passive transport processes which can be used as transport agents include but are not limited to lipophilic entities, polycations such as polylysine or oligolysine, other polyamines, etc. Compounds subject to receptor-mediated endocytosis which can be used include but are not limited to transferrin, epidermal growth

factor, and others known in the art. The transport agent may be selected from the group including but not limited to cholesterol, a peptide, a protein, a lipid (e.g. a fatty acid containing at least 12 carbon atoms, a triglyceride, a phospholipid, a glucocorticoid), a saccharide (e.g., mannose, glucose, galactose), an antibody, a nucleoside or nucleoside analog, and a biocompatible polymer (e.g. cellulose, polyethylene glycol, polyvinyl alcohol). In a specific embodiment, the transport agent is a fatty acid containing 18 carbon atoms. In another specific embodiment, the transport agent is cholesterol. A particular example of such a compound has the structure: cholesterol-dinucleotide-R_1-S-S-R_2-oligonucleotide, where R_1 and R_2 are hydrocarbon chains, and R_1 may be identical to R_2. In a particular aspect, the oligonucleotide is conjugated at its 5' terminus, via a disulfide linker, to cholesterol.

In one embodiment, the conjugates of the invention comprise a transport agent which facilitates passage through the blood-brain barrier. Since the blood-brain barrier primarily comprises lipids, in this embodiment, it is preferred that the transport agent be lipophilic. Such transport agents include but are not limited to cholesterol, a hydrophobic peptide, a fatty acid comprising at least 12 carbon atoms, a triglyceride, and a biocompatible polymer (e.g. cellulose, polyethylene glycol, polyvinyl alcohol). In another aspect of this embodiment, the transport agent can be a peptide capable of crossing the blood-brain barrier, such as one of those disclosed in PCT International Publication No. WO 89/10134, published November 2, 1989.

The above-recited transport agents may be modified at any position on their structure with substituents generally used in the art. Peptides, proteins, cholesterol, and lipid analogs may be

substituted with substituents including but not limited to alkyl, cycloalkyl, aryl, alkaryl, hydroxyalkyl, ester, ether, amide, halo, nitro, cyano, and carboxylic acid.

In addition, derivatized nucleotides or nucleotide analogs providing for hybridization-triggered cross-linking to other nucleotide sequences may be incorporated into conjugated oligonucleotides of the invention.

5.4. USES OF THE OLIGONUCLEOTIDE-TRANSPORT AGENT CONJUGATES OF THE INVENTION

1. Inhibition of Expression

The present invention relates to methods for inhibiting the expression of a nucleic acid sequence in a procaryotic or eucaryotic plant or animal cell comprising providing a viable cell in culture or *in vivo* with a composition comprising an effective amount of the oligonucleotide conjugates of the invention. In one embodiment, the expression of the nucleic acid sequence in the cell is inhibited by hybridization of the oligonucleotide with the nucleic acid sequence in the cell. In another embodiment, such composition may inhibit the expression of a nucleic acid in a cell by inhibiting the action of polymerases in the cell. In yet another embodiment, such composition may inhibit the expression of a nucleic acid sequence in a cell by forming a triple helix with a double-stranded nucleic acid sequence in the cell.

The nucleic acid sequence may be present in a procaryotic or eucaryotic cell, a normal or neoplastic cell. In a preferred embodiment, the cell is a mammalian cell. The nucleic acid sequence may be endogenous to the cell, or may be found within the cell yet specific to a pathogenic organism. Furthermore, the nucleic acid sequence may be a DNA or RNA sequence.

The compositions of the present invention may be useful as therapeutic agents, and for example may be used to inhibit the expression of bacterial or viral or fungal proteins, or of cellular proteins such as oncogenes, as well as T cell receptors, which are postulated to play a role in autoimmune diseases. Alternatively, the compositions may be useful for agricultural purposes. For example, such compositions may be used to alter the phenotypic characteristics of a plant, such as the modification of a particular enzymatic activity.

In a most preferred aspect, the oligonucleotide is cleaved from the transport agent during or after entry into a cell.

In one embodiment, the expression of a nucleic acid sequence may be inhibited by providing the cell with an effective amount of a composition comprising the oligonucleotide conjugates of the invention, in which the oligonucleotide portion of the conjugate consists of 6-50 nucleotides, with a size of 8-30 nucleotides most preferred, and is capable of hybridizing to at least a portion of the nucleic acid sequence. In this embodiment, the oligonucleotide sequence is "antisense" or complementary, and thus capable of hybridizing, to the nucleic acid sequence. In various aspects, the nucleic acid sequence can be contained within a single stranded, double stranded, or multiply stranded nucleic acid. Where the oligonucleotide conjugate binds to the oligonucleotide's complementary sequence contained on a double stranded nucleic acid, a triple helix can be formed.

In a specific aspect directed toward inhibiting the expression of a nucleic acid sequence, if the nucleic acid sequence is contained within a sequence of double stranded DNA, the oligonucleotide sequence may be complementary to the sequence of the complementary strand of the nucleic acid sequence. In

another sp cific aspect, the oligonucleotide sequence can be complementary to the RNA transcribed off the nucleic acid sequence.

In an alternative embodiment directed toward inhibition of nucleic acid sequence expression, the oligonucleotide of the conjugate need not have a sequence complementary to the nucleic acid sequence or to its complementary strand, since, e.g., certain oligonucleotides which are of random sequence or homopolymeric may inhibit the expression of certain non-complementary nucleic acid sequences such as those which are of viral origin (see e.g., Aradi, J. and Ho, Y. K., 1985, Cancer Biochem. Biophys. 7:349-359; Majumdar, C., et al., 1989, Biochem. 28(3):1340-1346).

In a specific embodiment, the expression of a nucleic acid sequence of a pathogenic organism can be inhibited. In a particular aspect, the oligonucleotide portion of the conjugates of the invention comprises a sequence complementary and capable of hybridizing to at least a portion of a DNA or RNA sequence of the pathogenic organism.

2. Therapeutic Applications

A variety of diseases and disorders can be treated by administration to a subject of a composition comprising an effective amount of the oligonucleotide conjugates of the invention. In a preferred aspect, once the composition is taken up by the cell, the disulfide linkage is cleaved and the oligonucleotide is released. Viral diseases and disorders which can be treated by administration of a conjugate of the invention, in which the oligonucleotide inhibits expression of a viral nucleic acid sequence, include but are not limited to those caused by hepatitis B virus, cytomegalovirus, herpes simplex virus I or II, human immunodeficiency virus type I or II, influenza virus, respiratory syncytial virus, and human papilloma virus. Malignancies which can be treated by adminis-

tration of a conjugate of the invention include but are not limited to lung cancer (e.g., small cell lung carcinoma), colorectal cancer, prostate cancer, breast cancer, leukemias and lymphomas. For example, in the treatment of a malignancy, the oligonucleotide portion of the conjugate can be complementary to (and capable of hybridizing to) a gene encoding an aberrantly expressed oncogene, or to a gene encoding a growth factor required for maintenance of the malignant state.

In a specific embodiment, where the transport agent facilitates crossing of the blood-brain barrier, the compositions may be used to treat a neurological disorder.

These disorders can also be detected by detecting nucleic acid sequences associated with the presence of such diseases, disorders or malignancies, as provided by the present invention.

3. Pharmaceutical Applications

For therapeutic use, pharmaceutical compositions are provided, consisting of an effective amount of the oligonucleotide conjugates of the invention formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA, latest edition.

For systemic administration, injection is preferred and may be intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the compositions of the invention are formulated in liquid solutions, such as deionized water, water, phosphate-buffered saline, or ethanol, and preferably in physiologically compatible buffers, such as Hank's or Ringer's. In addition, the compositions may be formulated in solid form and redissolved or suspended

immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the compositions may be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays, for example, or using suppositories. For oral administration, the compositions are formulated into conventional oral administration forms such as capsules, tablets, and tonics.

For topical administration, the compositions of the invention are formulated into ointments, salves, gels, or creams, as generally known in the art. Alternatively, the compositions may be formulated in the lumen of vesicles, such as liposomes.

In a particular aspect, an oligonucleotide conjugated at its 5' terminus to a transport agent via a molecular linker containing a disulfide, and which is endcapped at its 3' end with methoxyethylamine to help prevent degradation in vivo, is administered intravenously.

The compositions may be administered to a plant using various procedures known in the art (see Shewmaker et al., U.S. Patent No. 4,801,540, issued January 3, 1989). For example, the compositions of the present invention may be introduced into a suitable vector and administered to the plant via electroporation, transformation, inoculation, and the like.

4. Diagnostic and Detection Applications

An effective amount of an oligonucleotide conjugate of the invention in which the oligonucleotide

consists of at least 6 nucl otides, is capable of hybridizing to at least a portion of a nucleic acid sequence within a cell, and is detectably labeled, may be used as a detection or diagnostic agent by hybridizing to its complementary nucleic acid sequence within the cell. In this embodiment, an effective amount of such an oligonucleotide conjugate may be used to detect a nucleic acid sequence in a viable procaryotic or eucaryotic cell in culture or *in vivo*

The detectable label which is linked to the oligonucleotide may be selected from the group including but not limited to a radioactive group, an enzyme, a fluorescent group, and an antibody. Upon entry into the cell, the labelled oligonucleotide hybridizes to its complementary nucleic acid sequence within the cell, and is detected using procedures known in the art.

The present invention also includes methods for detecting the presence of a nucleic acid sequence of an exogenous infectious agent or of a selected gene utilizing oligonucleotide-disulfide conjugates as nucleic acid-based diagnostic probes.

One such method consists of an indirect assay, whereby human body fluids, tissue or cell extracts are screened for the presence of a target DNA by binding an oligonucleotide bound to a support membrane. An oligonucleotide probe containing a reporter group linked to an oligonucleotide via a disulfide linker can be added to a DNA mixture containing the target DNA sequence. The reporter group which may consist of, but is not limited to, an enzyme such as alkaline phosphatase may be released from the target DNA/oligonucleotide probe by adding a reducing reagent, e.g., dithiothreitol. The presence of target DNA can then be qualitatively and quantitatively measured by detection of the reporter group, e.g., spectrophotometrically.

Another such embodiment of the invention utilizes a diagnostic assay whereby an oligonucleotide probe containing a linker with a free thiol group can be used to hybridize with a target DNA sequence that has previously been immobilized on a solid support membrane. The resulting unbound DNA probe is then washed away and a reporter group containing a free thiol group is attached to the free thiol group of the oligonucleotide linker complex by disulfide bond formation. The presence of target DNA can then be measured by detection of the reporter group. In an alternative embodiment the oligonucleotide, rather than the target DNA, can be immobilized on the solid support.

In a specific embodiment, where disulfide linkers are incorporated into probes, nucleotides containing specific hybridization-triggered crosslinking agents can be incorporated within the oligonucleotide probe, in order to amplify sensitivity and reduce background in diagnostic assays. The use of crosslinking agents will permit novel diagnostic assay modifications such as a) the use of the crosslinker to increase probe discrimination b) incorporation of a denaturing wash step to reduce background and c) carrying out hybridization and crosslinking at or near the melting temperature of the hybrid DNA will reduce secondary structure in the target DNA and to increase probe specificity.

Another specific embodiment of the present invention involves using probes containing disulfide linkers in combination with base analogs such as 5 methycytosine, for use in diagnostic assays that are based on sequence specific binding to double stranded DNA. Such base analogs facilitate formation of triple helix structures by hybridization to DNA, thus circumventing the requirement for isolation of RNA, which is extremely laborious and sensitive to

ubiquitous nucleases. Additionally, such triple helix probes may be conjugated to various reporter groups known in the art to facilitate detection or quantitation of specific regions of double stranded DNA. Probes that form triple helix structures can also incorporate additional modifications such as altered internucleotide linkages that render the oligonucleotide nuclease stable. Such stable oligonucleotides will be useful for assays conducted in the presence of cell or tissue extracts which normally contain nuclease activity.

An additional embodiment of the present invention comprises using oligonucleotides with a thiol group for attachment to a solid support derivatized with thiol groups, by formation of a disulfide linkage, in order to bind complementary DNA sequences found in human body fluids, cell or tissue extracts. In this specific embodiment, the solid support is comprised of one of the following: Sulfolink® Coupling Gel Columns, Tresyl Activated Agarose, ImmunoPure® Epoxy-activated Agarose, and TNB Thiol Agarose (Pierce Chemical Company).

Another embodiment of this invention is comprised of double stranded oligonucleotides containing the binding site sequence recognized by a cognate DNA-binding protein, which can be used in the assays described above to bind specifically to a DNA-binding protein from a mixture of proteins isolated from cell or tissue extracts.

The following example is presented by way of illustration, not by way of limitation.

6. EXAMPLE: PREPARATION AND ASSAYS OF CHOLESTEROL-TC-R-S-S-R-CAGTGATT AND CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTTTCTCCAT

In the example described herein, the preparation of two oligonucleotides linked to cholesterol via a disulfide linkage, CHOLESTEROL-TC-R-S-S-R-CAGTGATT (I) and CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTTTCTCCAT (II), where -R-S-S-R- = $O_3PCH_2CH_2S$-$SCH_2CH_2PO_3$, and TC represents thymine and cytosine nucleotides (and A=adenine nucleotide; G=guanine nucleotide). Results from reduction analysis studies performed with compound II indicated that the disulfide linkage may be cleaved with a reducing agent. Uptake and serum stability studies performed using compound II indicated that compound II may be taken up by cells and is stable. The disulfide linkage is cleaved once compound II is taken up by the cell.

6.1. PREPARATION OF 2-DIMETHOXYTRITYL-2-HYDROXYETHYL DISULFIDE HYDROGEN PHOSPHONATE

2-Dimethoxytrityl-2-hydroxyethyl disulfide hydrogen phosphonate was prepared by reacting 2-dimethoxytrityl-2-hydroxyethyl disulfide with 2-chloro-4H-1,2,3-benzodioxaphosphorin-4-one.

2-Dimethoxytrityl-2-hydroxyethyl disulfide was prepared in the following manner. 2-Hydroxyethyl disulfide (2.5 g, 16.2 mmoles) was added to a 0°C solution of methylene chloride (50 ml) containing triethylamine (10 ml). 0.15 g N,N-dimethylamino-pyridine and 6.5 g (19.4 mmole) 4,4'-dimethoxytrityl chloride was added. After 2 hours at 0°C, the reaction was washed with a saturated sodium bicarbonate solution, dried over sodium sulfate and concentrated to dryness. The residue was purified by flash chromatography (Still et al., 1978, J. Org. Chem. 43:2923-2925) eluting with 1% triethylamine in methylene chloride,

producing 2-dimethoxytrityl-2-hydroxy thyl disulfide hydrogen phosphonate (2.7 g, 37% yield) as an oil.

2.3 g (15 mmoles) of 2-dimethoxytrityl-2-hydroxyethyl disulfide was added to a cold solution of 2-chloro-4H-1,2,3-benzodioxaphosphorin-4-one (15 ml of 1 M solution in methylene chloride, 15 mmoles). After two hours, the reaction was washed with 1 M triethylammonium bicarbonate (TEAB) solution. The organic solution was separated using a separator funnel, dried over sodium sulfate, and the solvent removed in vacuo. The residue was purified by flash chromatography using silica gel eluting with 2% triethylamine/methylene chloride, and 2% triethylamine/5% methanol/methylene chloride. The product fraction was washed with 1 M TEAB solution, dried over sodium sulfate and the solvent was removed to yield 1.8 g 2-dimethoxytrityl-2-hydroxyethyl disulfide hydrogen phosphonate (58% yield).

6.2. PREPARATION OF CHOLESTERYL HYDROGEN PHOSPHONATE TRIETHYLAMMONIUM SALT

Cholesterol (3.4 g; 8.8 mmole) in 50 ml methylene chloride was added to a 0°C methylene chloride solution (100 ml) or 2-chloro-4H-1,2,3-benzodioxaphozphorin-4-one (20 mmole in 20 ml 1 M methylene chloride) and pyridine (1.6. ml, 20mmole). After 30 min., the reaction was poured into 1 M triethylammonium bicarbonate (TEAB). The organic solution was separated, washed with 1 M TEAB, dried over sodium sulfate, and concentrated. The residue was purified by flash column chromatography, eluted with 10% methanol in methylene chloride and 15% methanol in methylene chloride. Fractions of product were washed with 1 M TEAB, dried, and concentrated, affording 1.05 g of the cholesteryl hydrogen-phosphonate triethylammonium salt in the form of a whit solid (21.6% yield).

6.3. PREPARATION OF CHOLESTEROL -TC-R-S-S-R-CAGTGATT (I)

The hydrogen phosphonate was incorporated into the 5' position of d-CAGTGATT using standard hydrogen phosphonate chemistry on a Biosearch automated DNA synthesizer using the procedure described by Froehler et al. (1986, Nucl. Acids Res. 14:5399-5407). The dimethoxytrityl group was removed and TC was subsequently added to R-S-S-R-CAGTGATT using standard hydrogen phosphonate chemistry on a Biosearch automated DNA synthesizer as described. TC-R-S-S-R-CAGTGATT was subsequently reacted with the triethylammonium salt of cholesteryl hydrogen-phosphonate triethylammonium salt. The structure of the reaction product is shown in Figure 1.

6.4. IN VITRO REDUCTION OF CHOLESTEROL -TC-R-S-S-R-CAGTGATT (I)

CHOLESTEROL-TC-R-S-S-R-CAGTGATT (I) was labelled at the 3' end by the incorporation of 10 μCi α-^{32}P-UTP (Amersham, 3000 Ci/mmole) using 10 U terminal transferase (New England Nuclear). The reaction mixture was incubated for 1 hour at 37°C using terminal transferase tailing buffer. The reaction mixture was subsequently diluted in 10 mM Tris, 1 mM EDTA, pH 7.5.

50,000 cpm of 3'-^{32}P-CHOLESTEROL-TC-R-S-S-R-CAGTGATT was treated with 1 and 10 mM dithiothreitol (DTT) under physiological conditions: 10 mM $MgCl_2$, 100 mM NaCl in 50 mM Tris, pH 7.5 at about 37°C for one hour. Deoxy-CAGTGATT was treated with 1 and 10 mM DTT to control for potential modification. The reaction was then diluted with an equal volume of 7 M urea loading buffer (20% sucrose, 0.1% xylene cyanol, 0.1% bromophenol blue, 0.1X TBE buffer) and analyzed on a 20%/8 M urea polyacrylamide gel. Bands were visualized via autoradiography at -70°C using an intensifying screen.

The results are shown in the "R" lane of Figure 2. The results indicate that quantitative cleavage of the disulfide linkage occurs when the oligonucleotide is treated with 10 mM DTT.

6.5. PREPARATION OF INTERNALLY ^{32}P-LABELLED CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTTTCTCCAT

The oligodeoxyribonucleotide 5'-TTTTTTTCTCCAT-3' was 5' end labelled using τ-^{32}P-ATP and T4 polynucleotide kinase. This oligonucleotide contains methoxyethylamine end-caps at the 2-3' most diester linkages and was prepared via hydrogen-phosphonate chemistry (Froehler, 1986, Tet. Letters 27:5575-5578). The oligodeoxyribonucleotide was phosphorylated at the 5' end by reacting the oligodeoxyribonucleotide with γ-^{32}P-ATP (ICN; 7000 Ci/mmole) and T4 polynucleotide kinase using the procedure of Maniatis et al. (Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, p. 122). After phosphorylation, the sample was heated at 65°C for 15 minutes. A 10-fold excess of template 5'-TGGCTGATGGAGAAAAAAATCACTGGAG ACCTC-3' and a 100 fold excess of CHOLESTEROL-TC-R-S-S-R-CAGTGATT was added to the solution. This mixture was heated to 85°C for 3 minutes and allowed to cool over 1 hour to 15°C. 10 U T4 DNA ligase and ATP (1 mM final concentration) were added and the ligation reaction was continued overnight at 15°C. The ligated product was purified via 15%/8 M urea polyacrylamide gel electrophoresis. Product was visualized via autoradiography and cut out of the gel. The ^{32}P-labelled oligonucleotide was eluted from the gel in 10 mM Tris, pH 7.5, 1 mM EDTA overnight. The sample was desalted and concentrated using a C8 quick sep column (Baker), eluting the ^{32}P-labelled oligodeoxyribonucleotide with 30% acetonitrile/water.

The solvent was then removed and the product resuspended in 100 μl of sterile water.

6.6. H938 UPTAKE AND REDUCTION ANALYSIS STUDY OF INTERNALLY ^{32}P-LABELLED CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTTTCTCCAT

300 μl of H938 cells (3 X 10^5 cells/ml) were incubated with ^{32}P-labelled CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTTTCTCCAT (4 X 10^6 cpm) which was endcapped at the 3' end with methoxy ethylamine (MEA) phosphoramidate in 10% heat inactivated fetal calf serum (FCS)-RPMI medium. At 0, 4, and 48 hours, 100 μl of cell suspension was removed and subjected to the fractionation protocol described below.

100 μl of cells were pelleted in an eppendorf microcentrifuge at 6000 rpm for 5 minutes at 4°C. 5 μl of the supernatant was removed for electrophoretic analysis. The pellet was resuspended in phosphate buffered saline (PBS) and 10 U DNase I was added. Digestion was then performed at 37°C for 10 minutes. The reaction was stopped by the addition of 5 mM EDTA. The cell suspension was pelleted via centrifugation in an eppendorf microcentrifuge at 6000 rpm for 5 minutes at 4°C. The cell pellet was washed with 100 μl PBS and respun in an eppendorf microcentrifuge at 6000 rpm for 5 minutes at 4°C. This procedure was repeated three times. The cell pellet was resuspended in 20 μl 10 mM Tris, pH 7.5 containing 15 mM KCl, 2 mM MgC12, 0.1 mM EDTA and 0.2% NP-40. The sample was placed on ice for 1 minute followed by 30 seconds of vortexing. This was repeated. This extract was then spun in an eppendorf microcentrifuge at 3000 rpm for 5 minutes at 4°C. The supernatant was then removed and diluted into 7 M urea loading buffer described in Section 6.3 supra. The pelleted nuclei were then disrupted in 20 μl of 10 mM Tris, pH 7.5 containing 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM

EDTA and 100 mM NaCl at 65°C for 5 minutes. An equal volume of 7 M urea loading buffer was then added.

The samples from this fractionation were then analyzed via electrophoresis using a 20% polyacrylamide/8 M urea gel. Bands were visualized via autoradiography at -70°C using an intensifying screen. As shown in Figure 2, the linkage was observed to be reduced in oligonucleotide reduced from cytoplasmic and nuclear fractions. No reduction was observed in oligonucleotide from the medium of the cell cultures (see Figure 3).

6.7. SERUM STABILITY OF ^{32}P-LABELLED CHOLESTEROL -TC-R-S-S-R-CAGTGATTTTTTTTTCTCCAT

3'-MEA endcapped internal ^{32}P-labelled CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTTTCTCCAT was tested for nuclease and reduction stability in RPMI medium supplemented with 15% heat inactivated FCS. 3'-MEA endcapped internal ^{32}P-labelled CHOLESTEROL-TC-R-S-S-R-CAGTGATTTTTTTTTCTCCAT (4 X 10^5 cpm) was added to 20 µl of cell medium. Five µl was removed at 0, 30, 60, and 180 minutes. This was diluted into 5 µl 7 M urea loading buffer and stored on ice. Samples were then analyzed using a 20% polyacrylamide/8 M urea gel. Bands were visualized via autoradiography at -70°C using an intensifying screen. As shown in Figure 4, the disulfide linkage is stable to reduction in media containing FCS.

7. EXAMPLE: PREPARATION OF 5'CAGCAGCXGCAGCAGCAGCAG 3' AND 5'CAGCAGCXGC 3'

This example describes the preparation of two oligonucleotides where an intranucleotide position has been changed to a disulfide analog where $X=O_3POCH_2CH_2OCH_2CH_2SSCH_2CH_2OCH_2CH_2OPO_3$. Results indicate this species is reducibly cleaved. Uptake and serum stability studies have been performed and indicat that

this analog is taken up by cells and is stable. Once intracellularly localized this linkag is cleaved.

7.1. PREPARATION OF (HYDROXYETHOXY)ETHANE THIOL

To a stirring solution of (hydroxyethoxy)ethyl chloride (10g;80 mmoles) in water was added 9.1g (120 mmoles) thiourea. A condenser was affixed and the material brought to reflux overnight under argon. At this time the reaction was cooled to room temperature and 100 mL (500 mmoles) 5N NaOH was added. The reaction was again brought to reflux for 3 hours. The solution was allowed to cool and brought to pH 4 via careful addition of conc.HCl under argon. An oil formed at this time which was separated from the aqueous phase. The aqueous layer was extracted 3 times with $CHCl_3$. The organic material pooled (including the initial oil) and extracted with saturated salt solution. The organic fraction was dried over sodium sulfate, filtered and the solvent removed under reduced pressure to leave hydroxyethoxyethane thiol as an oil. (7.8g; 80% yield).

7.2 PREPARATION OF (HYDROXYETHOXY)ETHYL DISULFIDE

3.75g (30.8 mmoles) (hydroxyethoxy)ethane thiol was dissolved in 1N NaOH (33mL;33mmoles). The solution was cooled to 0°C with a wet ice bath. 3.12g (12.32 mmoles) of solid iodine was added slowly over 90 minutes; small portions were added and the iodine color allowed to dissipate before a subsequent addition was made. Upon completion of iodine addition, the reaction was allowed to stir for 3 hours on ice, followed by warming to room temperature. This mixture was extracted 3 times with $CHCL_3$. The organic layers were pooled, extracted with saturated salt solution, and dried over sodium sulfate. The solvent was removed

under reduced pressure to yield (hydroxyethoxy)ethyl disulfide as clear viscous oil (2.22g; 50% yield).

7.3. PREPARATION OF MONO-(2-DIMETHOXYTRITYL)-(HYDROXYETHOXY)ETHYL DISULFIDE

211mg (0.87mmoles)(hydroxethoxy)ethyl disulfide was resuspended in 5mL of pyridine and the solvent removed under reduced pressure. This was repeated 2 times. The disulfide was then resuspended in 5mL pyridine, a magnetic stirring bar was added and the solution was set stirring. 294mg (0.87 mmoles) 2-dimethoxytrityl chloride was then added and the reaction monitored via tlc (solvent system: 5% isopropanol/methylene chloride/1% TEA. Upon completion the reaction was quenched with 1 mL methanol and allowed to stir for 30 minutes. The solvent was removed under reduced pressure. The oily solid was resuspended in 25mL of methylene chloride, extracted 3 times with water, once with saturated salt solution, and dried over sodium sulfate. The solvent was removed under reduced pressure and the protected disulfide purified via flash chromatography on silica gel using a gradient of 0-5% isopropanol in methylene chloride with 1% TEA. Yield-224mg (0.41 mmoles)/47% of mono-(2-dimethoxytrityl)-(hydroxyethoxy)ethyl disulfide.

7.4. PREPARATION OF MONO-(2-DIMETHOXYTRITYL)-(HYDROXYETHOXY)ETHYL DISULFIDE HYDROGEN PHOSPHONATE

49mg (0.09 mmoles) mono-(2-dimethoxytrityl)-(hydroxyethoxy) ethyl disulfide (1) was resuspended in 5 ml of pyridine and the solvent removed under reduced pressure.

1 was resuspended in 2 mL methylene chloride. This solution was added to a stirring solution of 2-chloro-4H-1,2,3-benzodioxaphosphorin-4-one in (1:1) pyridine/methylene chloride (0.225 mmoles) cooled to

0°C. This was stirred for 30 minutes at which time 20mL of 1M TEAB pH 7.5 was added slowly. This was allowed to stir for 2 hours. The reaction was poured into 25 mL of 1M TEAB pH 7.5. The aqueous layer was extracted 3 times with methylene chloride; the solvent was removed at reduced pressure. Mono-(2-dimethoxytrityl)-(hydroxyethoxy)ethyl disulfide hydrogen phosphonate(2) was purified via flash chromatography on silica gel using an acetonitrile/water/TEA solvent system. Yield-38.9mg/0.0545 mmoles (61%).

7.5. PREPARATION OF CAGCAGCXGCAGCAGCAGCAG(III) AND CAGCAGCXGC(IV)

The hydrogen phosphonate (2) was incorporated into the 5' position of GC using standard hydrogen phosphonate chemistry on a Biosearch automated DNA synthesizer using the procedure described by Froehler et al. ((1986) Nucleic Acids Res. 14 5399-5407). The dimethoxytritryl group was subsequently removed and oligodeoxynucleotide synthesis continued. Oxidation to the phosphodiester was performed using CCl_4 as previously described.

7.6. PREPARATION OF INTERNALLY 32P-LABELLED CAGCAGCXGCAGCAGCAGCAG

Oligonucleotide 5'-AGCAGCAGCAT-3' was 5' end labeled using α-^{32}P-ATP and T4 polynucleotide kinase. After phosphorylation, the sample was heated at 65°C for 15 minutes. To this solution, 10-fold excess template and 100-fold excess CAGCAGCXGC was added. This mixture was heated to 85°C for 3 minutes and allowed to slowly cool over 1 hour to 4°C. 10 U T4 DNA ligase and ATP (1 mM final) were added and the ligation reaction continued overnight. The ligated product was purified via 15% denaturing polyacrylamide gel electrophoresis. Product was visualized via

autoradiography and cut out of the gel. The ^{32}P labeled oligonucleotide was eluted from the gel, desalted and concentrated using C8 reverse phase chromatography. The product was eluted using 30% acetonitrile in water. The solvent was then removed and the product resuspended in 100 uL of sterile water.

7.7. H 938 UPTAKE AND REDUCTION ANALYSIS STUDY OF DISULFIDE CONTAINING OLIGONUCLEOTIDE (III)

300 uL of H938 cell ($3x10^3$ cell/mL) was incubated with internal ^{32}P labeled 3'-MEA-encapped oligonucleotide (II) ($4x10^6$ cpm). At 0, 4 and 48 hours, 100 uL of cell suspension was removed and subjected the fractionation protocol described below.

100 uL of cells were pelleted in eppendorf microcentrifuge (6000 PRM) for 5 minutes @4°C. 5 uL of the supernatent was removed for electrophoretic analysis. The pellet was resuspended in phosphate buffer saline (PBS) and 10 DNase I was added. Digestion was then performed at 37°C for 10 minutes. The reaction was stopped by the addition of 5 mM EDTA. The cell suspension was pelleted via centrifugation (6K-5mins). The cell pellet was washed with 100 uL PBS and respun (6K-5mins). This procedure was repeated three times. The cell pellet was resuspended in 20 uL of 10 mM Tris pH 7.5 containing 15 mM KCl, 2 mM MgCL2, 0.1 mM EDTA and 0.2% NP-40. The sample was placed on ice for 1 minute followed by 30 seconds of vortexing. This was repeated. This extract was then spun at 3K for 5 mins at 4°C. The supernatent was then removed and diluted into 7M urea loading buffer. The pelleted nuclei were then disrupted in 20 uL of 10 mM Tris pH 7.5 containing 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA and 100 mM NaCl at 65°C for 5 mins. An equal volume of 7M urea loading buffer was then added.

The samples from this fractionation were then analyzed via electrophoresis using a 20% denaturing

polyacrylamide gel. Bands were visualized via autoradiography at -70°C using an intensifying screen.

7.8. SERUM STABILITY OF OLIGONUCLEOTIDE

3'-MEA endcapped internal ^{32}P-labeled (III) was tested for nuclease and reduction stability in 10% HI-FCS 2 uL ($4x10^5$ cpm) internal ^{32}P-labeled (II) was added to 2 uL of cell medium. 5 uL removed at 0, 0.5, 1, 3 and 24 hours. This was diluted into 5 uL 7M urea loading buffer and stored on ice. Samples were then analyzed using a 20% denaturing polyacrylamide gel. Bands were visualized via autoradiography at -70°C using an intensifying screen. Both oligonucleotides were found to have an extracellular $t_{1/2}$ > 24 hours, while cell associated oligonucleotides were reduced with a $t_{1/2} \leq 1$ hour.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference herein in their entireties.

WHAT IS CLAIMED IS:

1. An oligonucleotide conjugate comprising an oligonucleotide or analog thereof conjugated to an agent which facilitates transport across the outer membrane of a cell, which conjugation is via a molecular linker containing at least one disulfide bond.

2. The oligonucleotide conjugate of claim 1 in which the agent is capable of being passively transported across the cell membrane.

3. The oligonucleotide conjugate of claim 1 in which the agent is capable of being actively transported across the cell membrane.

4. The oligonucleotide conjugate of claim 1 in which the agent is capable of promoting fusion of the cell membrane.

5. The oligonucleotide conjugate of claim 1 in which the agent is cholesterol.

6. The oligonucleotide conjugate of claim 1 in which the transport agent is a nucleoside or nucleoside analog.

7. The oligonucleotide conjugate of claim 1 in which the transport agent is an antibody.

8. The oligonucleotide conjugate of claim 1 in which the transport agent is lipophilic.

9. The oligonucleotide conjugate of claim 8 in which the transport agent is a lipid selected from the group consisting f a fatty acid containing at least 12

carbon atoms, a triglyceride, a phospholipid, and a glucocorticoid.

10. The oligonucleotide conjugate of claim 1 in which the transport agent is a peptide or protein.

11. The oligonucleotide conjugate of claim 10 in which the protein is epidermal growth factor.

12. The oligonucleotide conjugate of claim 10 in which the protein is transferrin.

13. The oligonucleotide conjugate of claim 1 in which the transport agent is a polycation.

14. The oligonucleotide conjugate of claim 1 in which the transport agent is a saccharide.

15. The oligonucleotide conjugate of claim 14 in which the saccharide is selected from the group consisting of mannose, glucose and galactose.

16. The oligonucleotide conjugate of claim 1 in which the transport agent is a biocompatible polymer.

17. The oligonucleotide conjugate of claim 1 in which the disulfide bond is cleaved during or after transport across the membrane.

18. The oligonucleotide conjugate of claim 5 in which the disulfide bond is cleaved during or after transport across the membrane.

19. The oligonucleotide conjugate of claim 6 in which the disulfide bond is cleaved during or after transport across th membrane.

20. The oligonucleotide conjugate of claim 8 in which the disulfide bond is cleaved during or after transport across the membrane.

21. The oligonucleotide conjugate of claim 10 in which the disulfide bond is cleaved during or after transport across the membrane.

22. The oligonucleotide conjugate of claim 14 in which the disulfide bond is cleaved during or after transport across the membrane.

23. The oligonucleotide conjugate of claim 16 in which the disulfide bond is cleaved during or after transport across the membrane.

24. The oligonucleotide conjugate of claim 1 in which the oligonucleotide is DNA consisting of from 6-50 bases.

25. The oligonucleotide conjugate of claim 2 in which the oligonucleotide is DNA consisting of from 6-50 bases.

26. The oligonucleotide conjugate of claim 3 in which the oligonucleotide is DNA consisting of from 6-50 bases.

27. The oligonucleotide conjugate of claim 5 in which the oligonucleotide is DNA consisting of from 6-50 bases.

28. The oligonucleotide conjugate of claim 6 in which the oligonucleotide is DNA consisting of from 6-50 bases.

29. The oligonucleotide conjugate of claim 7 in which the oligonucleotide is DNA consisting of from 6-50 bases.

30. The oligonucleotide conjugate of claim 8 in which the oligonucleotide is DNA consisting of from 6-50 bases.

31. The oligonucleotide conjugate of claim 10 in which the oligonucleotide is DNA consisting of from 6-50 bases.

32. The oligonucleotide conjugate of claim 13 or 11 in which the oligonucleotide is DNA consisting of from 6-50 bases.

33. The oligonucleotide conjugate of claim 14 in which the oligonucleotide is DNA consisting of from 6-50 bases.

34. The oligonucleotide conjugate of claim 16 in which the oligonucleotide is DNA consisting of from 6-50 bases.

35. The oligonucleotide conjugate of claim 24 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell.

36. The oligonucleotide conjugate of claim 27 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell.

37. The oligonucleotide conjugate of claim 28 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequ nce within the cell.

38. The oligonucleotide conjugate of claim 29 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell.

39. The oligonucleotide conjugate of claim 30 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell.

40. The oligonucleotide conjugate of claim 31 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell.

41. The oligonucleotide conjugate of claim 32 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell.

42. The oligonucleotide conjugate of claim 33 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell.

43. The oligonucleotide conjugate of claim 34 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell.

44. The oligonucleotide conjugate of claim 35 in which the nucleic acid sequence is that of a pathogenic organism.

45. The oligonucleotide conjugate of claim 36 in which the nucleic acid sequence is that of a pathogenic organism.

46. The oligonucleotide conjugate of claim 35 in which the nucleic acid sequence is endogenous to the cell, and the cell is mammalian.

47. The oligonucleotide conjugate of claim 36 in which the nucleic acid sequence is endogenous to the cell, and the cell is mammalian.

48. An oligonucleotide conjugate comprising an oligonucleotide or analog thereof conjugated to an agent which facilitates transport across the blood-brain barrier within a mammal, which conjugation is via a molecular linker containing at least one disulfide bond.

49. The oligonucleotide conjugate of claim 48 in which the agent is lipophilic.

50. The oligonucleotide conjugate of claim 48 in which the agent is cholesterol.

51. The oligonucleotide conjugate of claim 48 in which the agent is a peptide.

52. The oligonucleotide conjugate of claim 48 in which the oligonucleotide is DNA consisting of from 6-50 bases.

53. The oligonucleotide conjugate of claim 52 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within a brain cell.

54. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 1 and a pharmaceutically acceptable carrier.

55. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 2 and a pharmaceutically acceptable carrier.

56. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 3 and a pharmaceutically acceptable carrier.

57. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 4 and a pharmaceutically acceptable carrier.

58. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 5 and a pharmaceutically acceptable carrier.

59. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 6 and a pharmaceutically acceptable carrier.

60. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 7 and a pharmaceutically acceptable carrier.

61. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 8 and a pharmaceutically acceptable carrier.

62. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 9 and a pharmaceutically acceptable carrier.

63. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 10 and a pharmaceutically acceptable carrier.

64. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 11 and a pharmaceutically acceptable carrier.

65. A pharmaceutical composition comprising an effectiv amount of the oligonucleotide conjugate of claim 12 and a pharmaceutically acceptable carrier.

66. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 13 and a pharmaceutically acceptable carrier.

67. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 14 and a pharmaceutically acceptable carrier.

68. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 15 and a pharmaceutically acceptable carrier.

69. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 16 and a pharmaceutically acceptable carrier.

70. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 17 and a pharmaceutically acceptable carrier.

71. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 24 and a pharmaceutically acceptable carrier.

72. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 25 and a pharmaceutically acceptable carrier.

73. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 26 and a pharmaceutically acceptable carrier.

74. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 27 and a pharmaceutically acceptable carrier.

75. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 28 and a pharmaceutically acceptable carrier.

76. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 29 and a pharmaceutically acceptable carrier.

77. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 30 and a pharmaceutically acceptable carrier.

78. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 31 and a pharmaceutically acceptable carrier.

79. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 32 and a pharmaceutically acceptable carrier.

80. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 33 and a pharmaceutically acceptable carrier.

81. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 34, 35 or 36 and a pharmaceutically acceptable carrier.

82. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 37, 38 or 39 and a pharmaceutically acceptable carrier.

83. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 40, 41 or 42 and a pharmaceutically acceptable carrier.

84. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 43 or 44 and a pharmaceutically acceptable carrier.

85. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 48 and a pharmaceutically acceptable carrier.

86. A method for inhibiting the expression of a nucleic acid sequence within a cell comprising providing the cell with an effective amount of the oligonucleotide conjugate of claim 1, 2 or 3.

87. A method for inhibiting the expression of a nucleic acid sequence within a cell comprising providing the cell with an effective amount of the oligonucleotide conjugate of claim 4, 5 or 17.

88. A method for inhibiting the expression of a nucleic acid sequence within a cell comprising providing the cell with an effective amount of the oligonucleotide conjugate of claim 24.

89. A method for inhibiting the expression of a nucleic acid sequence within a cell comprising providing the cell with an effective amount of the oligonucleotide conjugate of claim 25.

90. A method for inhibiting the expression of a nucleic acid sequence within a cell comprising

providing the cell with an effective amount of the oligonucleotide conjugate of claim 26.

91. A method for inhibiting the expression of a nucleic acid sequence within a cell comprising providing the cell with an effective amount of the oligonucleotide conjugate of claim 27.

92. A method for inhibiting the expression of a nucleic acid sequence within a cell comprising providing the cell with an effective amount of the oligonucleotide conjugate of claim 24, in which the oligonucleotide is capable of hybridizing to the nucleic acid sequence.

93. The method according to claim 92 in which the agent is passively transported across the cell membrane.

94. The method according to claim 92 in which the agent is actively transported across the cell membrane.

95. The method according to claim 92 in which the agent is cholesterol.

96. The method according to claim 92 in which the transport agent is lipophilic.

97. The method according to claim 92 in which the transport agent is a nucleoside or nucleoside analog.

98. The method according to claim 92 in which the transport agent is a peptide or protein.

99. The method according to claim 92 in which the transport agent is an antibody.

100. The method according to claim 92 in which the transport agent is a saccharide.

101. The method according to claim 92 in which the transport agent is a polycation.

102. The method according to claim 92 in which the transport agent is a biocompatible polymer.

103. The method according to claim 92 in which the disulfide bond is cleaved during or after transport across the membrane.

104. A method for detecting the presence of a nucleic acid sequence within a cell comprising providing a viable cell with the oligonucleotide conjugate of claim 24, 25 or 26, in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell, and the oligonucleotide is detectably labeled.

105. A method for detecting the presence of a nucleic acid sequence within a cell comprising providing a viable cell with the oligonucleotide conjugate of claim 27, 28 or 29, in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell, and the oligonucleotide is detectably labeled.

106. A method for detecting the presence of a nucleic acid sequence within a cell comprising providing a viable cell with the oligonucleotide conjugate of claim 30, 31 or 32 in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell, and the oligonucleotide is detectably labeled.

107. A method for detecting the presence of a nucleic acid sequence within a cell comprising providing a viable cell with the oligonucleotide conjugate of claim 33 or 34, in which the oligonucleotide is capable of hybridizing to a nucleic acid sequence within the cell, and the oligonucleotide is detectably labeled.

108. A method of treating a patient with a disease or disorder comprising administering the pharmaceutical composition of claim 54 to the patient.

109. A method of treating a patient with a disease or disorder comprising administering the pharmaceutical composition of claim 71 to the patient.

110. A method of treating a patient with a disease or disorder comprising administering the pharmaceutical composition of claim 74 to the patient.

111. A method of treating a patient with a disease or disorder comprising administering the pharmaceutical composition of claim 85 to the patient.

112. A compound having the formula: cholesterol-dinucleotide-R_1-S-S-R_2-oligonucleotide, in which R_1 and R_2 are hydrocarbon chains, and R_1 may be identical to R_2.

113. A pharmaceutical composition comprising an effective amount of the oligonucleotide conjugate of claim 41 and a pharmaceutically acceptable carrier.

114. A method for detecting the presence of a nucleic acid sequence within a cell comprising providing a viable cell with the oligonucleotide conjugate of claim 32 in which the oligonucleotide is

capable of hybridizing to a nucleic acid sequence within the cell, and the oligonucleotide is detectably labeled.

115. The oligonucleotide conjugate of claim 1 wherein the molecular linker has the formula:

$$Y\text{-}XCH_2CHR_1\text{-}SS\text{-}CHR_1CH_2X\text{-}Y$$

wherein:

X is O, $\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}$, $\overset{\overset{O}{\|}}{S}$, NR_1, CH_2, $C(R_1)_2$, or $\overset{\overset{O}{\|}}{C}$;

Y is H, alkyl, aryl or $\overset{\overset{O}{\|}}{C}$ when X is NR_1, CH_2 or $C(R_1)_2$; and

R_1 is H, CH_3, alkyl or aryl.

116. The molecular linker of claim 115 wherein:

X is O or NH_2;

Y is $-CH_2CH_2-$ or $\overset{\overset{O}{\|}}{C}$; and

R_1 is H or CH_3.

117. A method for isolating a nucleic acid sequence comprising:

a) binding a oligonucleotide via a linker comprising a disulfide bond to a solid support;

b) contacting the solid support attached to the oligonucleotide with a sample containing nucleic acid under conditions such that nucleic acid sequences in the sample can hybridize to the oligonucleotide;

c) washing to remove nucleic acid sequences in the sample that do not hybridize to the oligonucleotide;

d) cleaving the disulfide bond by exposure to reducing conditions; and

e) recovering the hybridized nucleic acid-oligonucleotide complex released by step (d).

118. A method for isolating a DNA binding protein comprising:

a) binding a double-stranded oligonucleotide via a linker comprising a disulfide bond to a solid support;

b) contacting the solid support attached to the oligonucleotide with a sample containing protein under conditions such that a DNA binding protein in the sample can bind to the oligonucleotide;

c) washing to remove protein in the sample that do not bind to the oligonucleotide;

d) cleaving the disulfide bond by exposure to reducing conditions; and

e) recovering the bound protein-oligonucleotide complex released by step (d).

119. A method for detecting a nucleic acid sequence comprising:

a) immobilizing DNA suspected of containing a nucleic acid sequence on a solid support;

b) hybridizing an oligonucleotide disulfide conjugate containing a reporter group to the DNA;

c) washing the solid support to remove oligonucleotide-disulfide conjugates that have not hybridized to the DNA;

d) cleaving the reporter group from the oligonucleotide-DNA complex by exposure to reducing conditions; and

e) detecting the reporter group, and thereby detecting the nucleic acid sequence.

120. A method for detecting a nucleic acid sequence in a sample comprising:

a) immobilizing an oligonucleotide containing a free thiol group on a solid support;

b) contacting the immobilized oligonucleotide with a sample containing nucleic acid under conditions such that the nucleic acid sequences in the sample can hybridize to the oligonucleotide;

c) washing the solid support to remove unhybridized nucleic acid;

d) contacting the solid support with a compound comprising a reporter group and containing a free thiol under oxidizing conditions such that a disulfide bond is formed between the free thiol on the oligonucleotide and the free thiol on the compound;

e) washing the solid support to remove any unbound compound; and

f) detecting any bound reporter group, and thereby detecting the nucleic acid sequence.

121. A method for detecting a nucleic acid sequence comprising:

a) immobilizing DNA on a solid support;

b) contacting the immobilized DNA with an oligonucleotide containing a free thiol group under conditions such that the DNA can hybridize to the oligonucleotide;

c) washing the solid support to remove unhybridized oligonucleotide;

d) contacting the solid support with a compound comprising a reporter gr up and containing a free thiol under oxidizing conditions such